Induction of Cytochrome P450 Enzymes and Generation of Protein-Aldehyde Adducts Are Associated With Sex-Dependent Sensitivity to Alcohol-Induced Liver Disease in Micropigs

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To assess possible links between ethanol-induced oxidant stress, expression of hepatic cytochrome P450 (CYP) enzymes, and sex steroid status, we used immunohistochemical methods to compare the generation of protein adducts of acetaldehyde (AA), malondialdehyde (MDA), and 4-hydroxynonenal (4-HNE) with the amounts of CYP2E1, CYP2A, and CYP3A in the livers of castrated and noncastrated male micropigs fed ethanol for 12 months. In castrated micropigs, ethanol feeding resulted in accumulation of fat, hepatocellular necrosis, inflammation, and centrilobular fibrosis, whereas only minimal histopathology was observed in their noncastrated counterparts. CYP2A and CYP3A were more prominent in the castrated animals than in the noncastrated micropigs. Ethanol feeding increased the hepatic content of all CYP forms. The most significant increases occurred in CYP2E1 and CYP3A in the noncastrated animals and in CYP2E1 and CYP2A in the castrated animals. Ethanol-fed castrated animals also showed the greatest abundance of perivenular adducts of AA, MDA, and HNE. In the noncastrated ethanol-fed micropigs a low expression of each CYP form was associated with scant evidence of aldehyde-protein adducts. Significant correlations emerged between the levels of different CYP forms, protein adducts, and plasma levels of sex steroids. The present findings indicate that the generation of protein-aldehyde adducts is associated with the induction of several cytochrome enzymes in a sex steroid-dependent manner. It appears that the premature, juvenile, metabolic phenotype, as induced by castration, favors liver damage. The present findings should be implicated in studies on the gender differences on the adverse effects of ethanol in the liver. (HEPATOLOGY 1999;30:1011-1017.)

Toxic effects of acetaldehyde (AA), the first metabolite of ethanol, and aldehydic products of lipid peroxidation have been suggested to play an important role in the pathogenesis of alcohol-induced liver disease.[1-3] Various reactive aldehydic products including AA and the oxidant products, malondialdehyde (MDA) and 4-hydroxynonenal (HNE), may be generated *in vivo* as a result of alcohol consumption. These metabolic products can bind to exposed proteins and cellular constituents forming stable adducts.[1,4-6] The appearance of aldehyde-protein adducts in zone 3 hepatocytes has been shown to be a typical feature of alcohol-induced liver disease.[5,7,8] Our previous follow-up studies in ethanol-fed micropigs have shown that the formation of aldehyde adducts in the liver coincide with elevation of serum transaminases and progressive histopathology.[7] High levels of reactive aldehydic products in the liver also appear to be associated with activation of fibrogenesis.[9]

Previous studies in rats intragastrically fed with ethanol have suggested a connection between enhanced lipid peroxidation and the induction of the ethanol-oxidizing enzyme cytochrome P450IIE1 (CYP2E1).[10-12] According to recent cell culture experiments and studies in ethanol-fed rats, heavy alcohol consumption may also induce other CYPs, such as CYP3A.[13-16] CYP2A induction by ethanol has not been previously examined, whereas both CYP2A and CYP3A have been shown to increase in mice with cocaine-induced liver disease.[17]

The present work was designed to compare the appearance and hepatocellular distribution of various aldehyde-protein adducts and CYP2E1, CYP2A, and CYP3A enzymes by immunohistochemistry of liver samples harvested from micropigs fed ethanol for 12 months. Because the susceptibility for the development of alcohol-induced liver disease is known to differ between males and females, the present experiments were performed in both castrated and noncastrated male micropigs and the results were correlated with serum testosterone and 17-β-estradiol (17-β-EST) hormone levels at the time of terminal liver biopsy.

MATERIALS AND METHODS

Animals. The micropig model for alcohol consumption has been previously described in detail.[18,19] These experimental animals consume ethanol voluntarily and completely in their daily diet. Normal intact or castrated 6-month-old male Yucatan micropigs, each weighing approximately 20 kg, were fed identical custom-blended Lab Micropig Chow Starter 5080-2 (Purina Mills, Richmond, IN), corn oil, and corn starch diets, as described.[18,19] Male micropigs were chosen for the study for convenience of animal care. Fat in the form of corn oil provided 33.6% of daily calories. Diets were provided and consumed at 90 kcal/kg body weight per day as a slurry in 3 equal rations at 8:00 AM, 12:00 PM, and 5:00 PM. Ethanol-fed groups of intact (n = 6) or castrated (n = 6) micropigs

Abbreviations: AA, acetaldehyde; MDA, malondialdehyde; 4-HNE, 4-hydroxynonenal; CYP, cytochrome P450; 17-β-EST, 17-β-estradiol; BSA, bovine serum albumin; LDL, low-density lipoprotein; PBS, phosphate-buffered saline; IgG, immunoglobulin G.

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received ethanol at 5 g/kg body weight daily, equivalent to 40% of total calories. Control groups of intact (n = 6) or castrated (n = 5) micropigs received identical diets, except that an isocaloric amount of corn starch was substituted for ethanol. The control subgroups were pair-fed to ethanol-fed subgroups within each larger group of intact or castrated animals. The micropigs were housed at the Animal Resource Service Facilities at the University of California Davis that are approved by the National Institutes of Health and were cared for according to the procedures outlined in "Guide for the Care and Use of Laboratory Animals," prepared by the National Academy of Sciences. Liver tissues were obtained at open laparotomy under anesthesia from castrated control and ethanol-fed micropigs at 1, 5, and 12 months of feeding and from intact control and ethanol-fed micropigs after 12 months of feeding. Details of feeding protocols and their biological effects have been reported previously.[7,18,19]

Antisera. Polyclonal rabbit antiserum against AA-modified epitopes was generated by immunizing rabbits with homologous AA–bovine serum albumin (BSA) as described.[20] Antibodies against acetaldehyde modified protein (AA1) were raised in rabbits using BSA conjugated with 1 mmol/L AA under reducing conditions as immunogen. The specificity of the antiserum has been shown earlier.[5,6] Polyclonal guinea-pig antisera against MDA and HNE adducts[21] were generous gifts of Dr. Seppo Ylä-Herttuala (University of Kuopio, Finland). Antisera against MDA–low-density lipoprotein (LDL) were raised by immunizing male guinea pigs with homologous MDA-LDL. The priming immunization was an intradermal injection of 150 μg of antigen in Freund's incomplete adjuvant at 14-day intervals. Conjugation of 4HNE to LDL was performed under reducing conditions, and polyvalent antisera were generated by immunizing male guinea pigs with homologous 4HNE-LDL. Monoclonal CYP2E1 (1-98-1) and CYP3A2 (2-13-1) antibodies were used for the detection of CYP2E1 and CYP3A.[17,22] A previously described chicken anti-CYP2A antibody was used for the detection of CYP2A.[17]

Liver Histology and Immunohistochemistry. Liver biopsy specimens were fixed in 4% neutral-buffered formaldehyde, dehydrated, and embedded in paraffin. For routine histological examinations, liver sections of 5 μm were stained with hematoxylin-eosin and with the Weigert van Gieson, or the Masson trichrome for collagen and were scored for the presence of fat, inflammation, necrosis, and fibrosis by 3 independent observers (ON, SP, and Dr. Boris Ruebner, University of California, Davis) using scoring scale between 0 (normal) to 4 (maximal change) for each parameter. For immunohistochemistry the sections were stained by the biotin-streptavidin complex method using the following steps: (1) pretreatment of the sections with undiluted cow colostral whey or swine serum for 40 minutes and rinsing in phosphate-buffered saline (PBS); (2) incubation for 1 hour with the primary antiserum diluted 1:200 (anti-CYP2A), 1:50 (anti-CYP3A and anti-CYP2E1), or 1:500 (antisera against AA, MDA, and HNE adducts) in 1% BSA-PBS; (3) treatment with cow colostral whey or swine serum for 40 minutes and rinsing in PBS; (4) incubation for 1 hour with biotinylated swine anti-rabbit immunoglobulin G (IgG) (Dakopatts, Glostrup, Denmark), goat anti-guinea pig IgG (Amersham International, Amersham, UK), goat anti-mouse IgG (Dakopatts), or rabbit anti-chicken IgG (Zymed Laboratories, San Francisco, CA) as appropriate, diluted 1:300 in 1% BSA-PBS; (5) incubation for 30 minutes with peroxidase-conjugated streptavidin (Dakopatts) diluted 1:500 in PBS; (6) incubation for 2 minutes in solution containing 9 mg 3,3′-diaminobenzidine tetrahydrochloride (DAB) (Fluka, Buchs, Switzerland) in 15 mL PBS plus 10 μL 30% H_2O_2. The sections were washed 3 times for 10 minutes in PBS after the incubation steps 2, 4, and 5. All the incubations and washings were performed at room temperature, and the sections were finally mounted in Permount (Fisher Scientific, Fair Lawn, NJ). The intensity of the staining was scored on a scale of 0 to +++ independently by 2 of the investigators (O.N. and S.P.) in a blinded fashion as follows: 0, no reaction; (+), scanty reaction; +, weak reaction; ++, moderate reaction; and +++, strong reaction. The stained sections were photographed with a Leitz Aristoplan microscope (Wetzlar, Germany). Digital image analyses were performed on an MCID-M2 image analyzer. Immunostaining reactions for AA, MDA, and HNE in the castrated micropigs have been reported previously.[7] These prior aldehyde immunostaining results and new aldehyde immunostainings were compared with the timing and intensity of cytochrome enzyme stainings in all animals included in the present series of experiments.

Other Methods. Testosterone and 17-β-EST concentrations were measured using conventional radioimmunological procedures.

Statistics. Values are expressed as mean ± SD. Testing for differences between indexed values in multiple groups was performed by Kruskall-Wallis test and in 2 groups by Mann-Whitney test. The differences were considered statistically significant at $P < .05$. Spearman's rank-correlation test was used to calculate correlations between the different variables. The abbreviation r_S stands for the correlation coefficients for Spearman's rank-correlation test.

RESULTS

Effects of Castration on Terminal Sex Steroid Levels. Among castrated micropigs, preterminal serum concentration of free testosterone were absent, whereas the levels of 17-β-EST were 10- to 100-fold decreased compared with the levels in the noncastrated animals (Table 1). Ethanol feeding significantly increased 17-β-EST levels in the castrated micropigs ($P < .01$), but had no significant effect on either 17-β-EST or testosterone levels in the noncastrated animals (Table 1).

Sequential Appearance of CYP Enzymes Compared With Liver Histology and Aldehyde Adducts. As previously reported,[7,18] ethanol feeding of castrated micropigs resulted in progressive histopathologic signs of alcohol-induced liver injury ranging from mild fatty change at 1 month to increased tissue damage including necrosis and fibrosis at 12 months (Fig. 1). Although the amount of fat remained relatively constant during the follow-up, the amount of inflammation at 5 months was significantly higher than that at 1 month ($P < .05$) In the noncastrated animals, there were no liver injury reactions to the ethanol treatment.[19] Parallel sections from these liver samples were used to compare the appearance of aldehyde adducts and CYP enzymes by immunohistochemistry. As previously described, AA adducts and oxidation-derived protein adducts, MDA, and HNE were present in acinar zone 3 hepatocytes of ethanol-fed castrated micropigs by 1 and 5 months.[7] The hepatic content of the immunodetectable CYP enzymes increased significantly as a result of ethanol feeding in castrated micropigs, such that the levels of each cytochrome enzyme were greatest at 12 months after the initiation of the ethanol feeding (Fig. 1A-C).

Figure 2 shows the typical distribution of the various hepatic CYPs characterized by immunohistochemistry in liver sections obtained at 1, 5, and 12 months after the

TABLE 1. Sex Steroids in Terminal Serum Samples From Castrated and Noncastrated Micropigs

	17-β-EST pg/mL	Testosterone ng/mL	17-β-EST/Testosterone $\times 10^{-3}$
Castrated animals			
Ethanol fed	62.4 ± 7.7	0	—
Control fed	12.6 ± 7.7*	0	—
Noncastrated animals			
Ethanol fed	624 ± 255	2.1 ± 0.8	514 ± 258
Control fed	1126 ± 443	7.7 ± 3.2	188 ± 63

*$P < .01$.

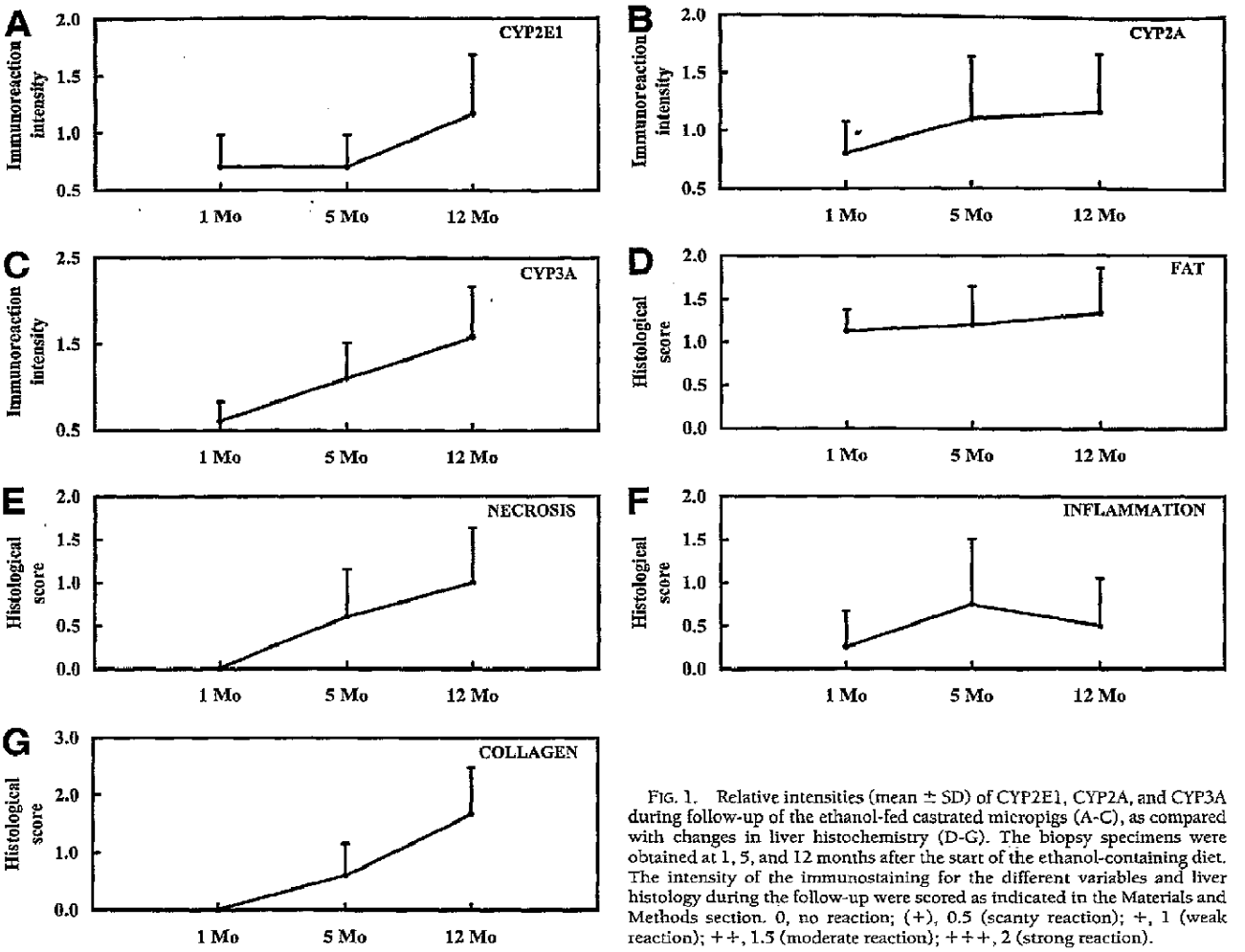

FIG. 1. Relative intensities (mean ± SD) of CYP2E1, CYP2A, and CYP3A during follow-up of the ethanol-fed castrated micropigs (A-C), as compared with changes in liver histochemistry (D-G). The biopsy specimens were obtained at 1, 5, and 12 months after the start of the ethanol-containing diet. The intensity of the immunostaining for the different variables and liver histology during the follow-up were scored as indicated in the Materials and Methods section. 0, no reaction; (+), 0.5 (scanty reaction); +, 1 (weak reaction); ++, 1.5 (moderate reaction); +++, 2 (strong reaction).

initiation of the ethanol feeding of castrated micropigs. At baseline, the expression of CYP2E1 and CYP2A was either low or absent (data not shown) and increased gradually after the start of the ethanol-containing diet. For both CYP2E1 (Fig. 2A-C) and CYP2A (Fig. 2D-F) the positive reactions were concentrated in zone 3 hepatocytes. The staining intensity and pattern varied between different CYPs, becoming gradually more intense and widespread in the hepatic lobule. The amount of CYP3A also increased during the ethanol diet (Fig. 2G-I). In the biopsy specimens obtained at 5 or 12 months (Fig. 2H and 2I), the most prominent immunoreactions continued to be localized in the perivenular region, whereas some signals extended to the zone 2 of the hepatic acinus. In control animals, only weak or no reaction was obtained (Fig. 2J-L), the strongest reaction being observed for CYP3A (Fig. 2L). No reaction was observed in stainings using nonimmune serum in place of the first antibody (data not shown).

Comparison of the Effects Between Castrated and Noncastrated Micropigs. Among castrated micropigs, the serum levels of free testosterone were nondetectable and levels of 17-β-EST were diminished but greater in the ethanol-fed group than the control group (Table 1). The blood ethanol levels were similar in the castrated and noncastrated animals and consistently exceeded 170 mg/dL after the last daily feeding.[18,19] Concurrent immunohistochemical stainings for the CYP enzymes and aldehyde-protein adducts were performed for both castrated and noncastrated micropigs from liver specimens obtained at 12 months after the initiation of the diet. The amounts and distributions of both aldehyde-protein adducts and CYP enzymes as well as liver histology findings were quite different between the control and ethanol-fed animals in the castrated and noncastrated groups (Fig. 3A-F). Except for CYP3A (Fig. 3C), the immunohistochemically detectable CYP enzymes were absent or very weak in the noncastrated micropigs fed the control diet, whereas the basal expression of both CYP2A and CYP3A were significantly enhanced in the castrated micropigs fed the control diet (Fig. 3B and C). The hepatic contents of CYP2A and CYP3A were each more abundant in the castrated animals fed either ethanol or control diets (Fig. 3B and C). Ethanol feeding increased CYP2E1 expression in both castrated and noncastrated animals (Fig. 3A), but ethanol feeding significantly increased the levels of CYP2A only in the castrated animals ($P < .05$, Fig. 3B). CYP3A was also induced by ethanol, although the differences reached significance only in the

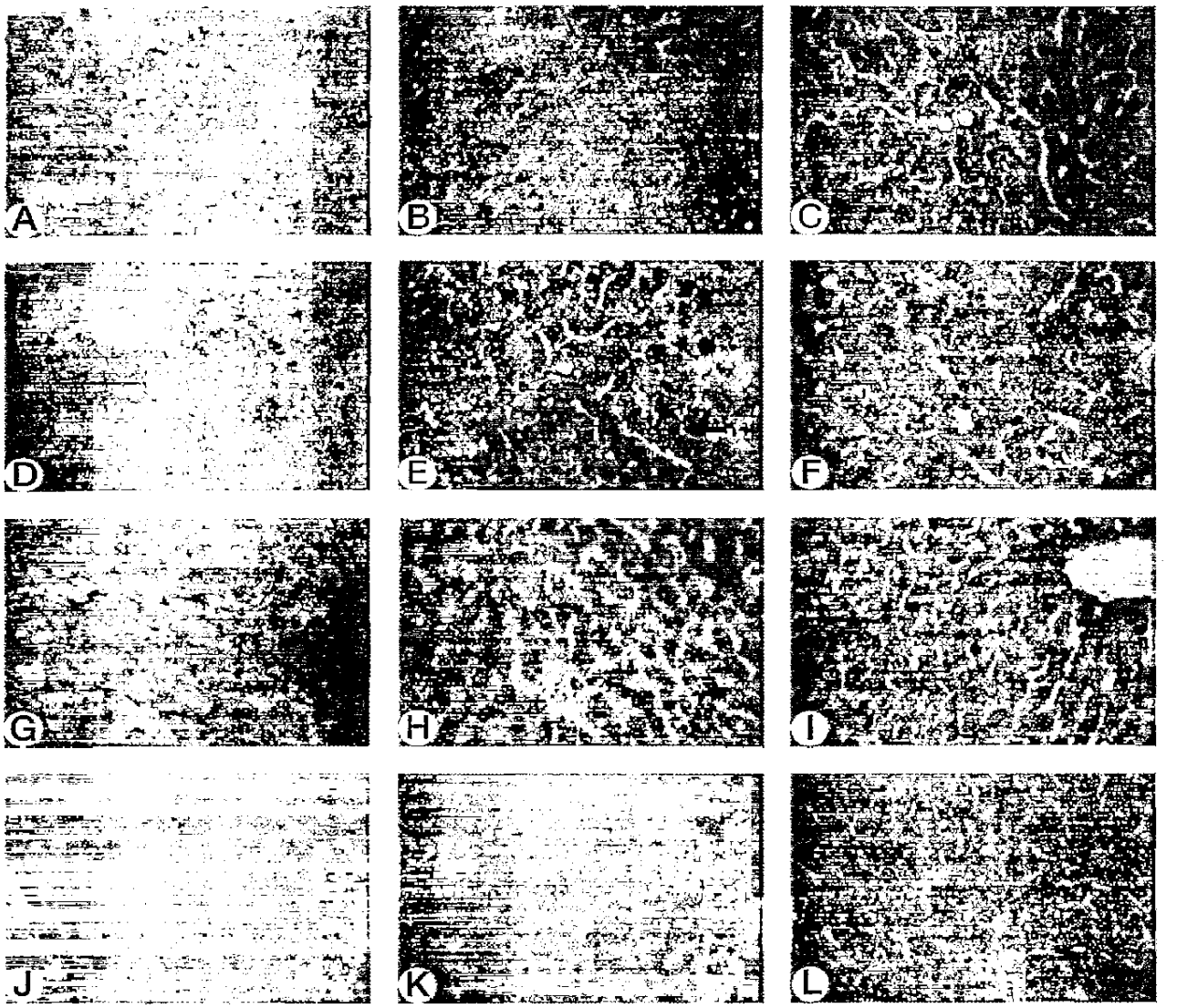

FIG. 2. Immunohistochemical staining of CYP2E1 (A-C), CYP2A (D-F), and CYP3A (G-I) in the liver of a castrated micropig fed with ethanol for 1 (A, D, G), 5 (B, E, H), and 12 months (C, F, I). Panels J-L show the stainings from a castrated control animal specimen immunostained with CYP2E1 (J), CYP2A (K), and CYP3A (L). Note the differences in the staining intensities between the various cytochromes. To allow comparisons between the data in Fig. 1, individual scores were as follows: A-C: 0.5, 1, 1.5; D-F: 1, 2, 1.5; G-I: 0.5, 1.5, 2, respectively. Original magnifications ×250.

noncastrated animals ($P < .05$, Fig. 3C). Aldehyde-protein adducts also increased more markedly in the castrated animals (Fig. 3D-F).

Correlations Between the Variables. Significant correlations were found among the cytochrome enzymes, protein adducts, and serum steroid hormone levels (Tables 2-4). Among noncastrated micropigs, only CYP2A correlated weakly with HNE and CYP3A correlated with AA adducts (Table 2). On the other hand, among castrated micropigs, CYP2A correlated with CYP3A, CYP2E1, AA, and MDA, whereas CYP2E1 correlated with both AA and MDA (Table 3). Among noncastrated animals, significant inverse correlations emerged between hepatic levels of CYP2E1 and serum levels of 17-β-EST and testosterone. Among castrated animals, both CYP2E1 and CYP2A correlated directly with serum levels of 17-β-EST (Table 4).

DISCUSSION

The present data suggest an interaction triad between hepatic cytochrome P450 content, oxidant stress, and sex hormone levels in experimental alcohol-induced liver disease. Hepatic CYP2E1 protein expression was induced by ethanol feeding and mainly expressed in zone 3 hepatocytes in alcohol-induced liver injury in both castrated and noncastrated animals. In addition, the present study provides the first experimental demonstration of increased hepatic CYP2A and CYP3A expression *in vivo* as a result of ethanol intake. The hepatic CYP2A and CYP3A levels were much greater in

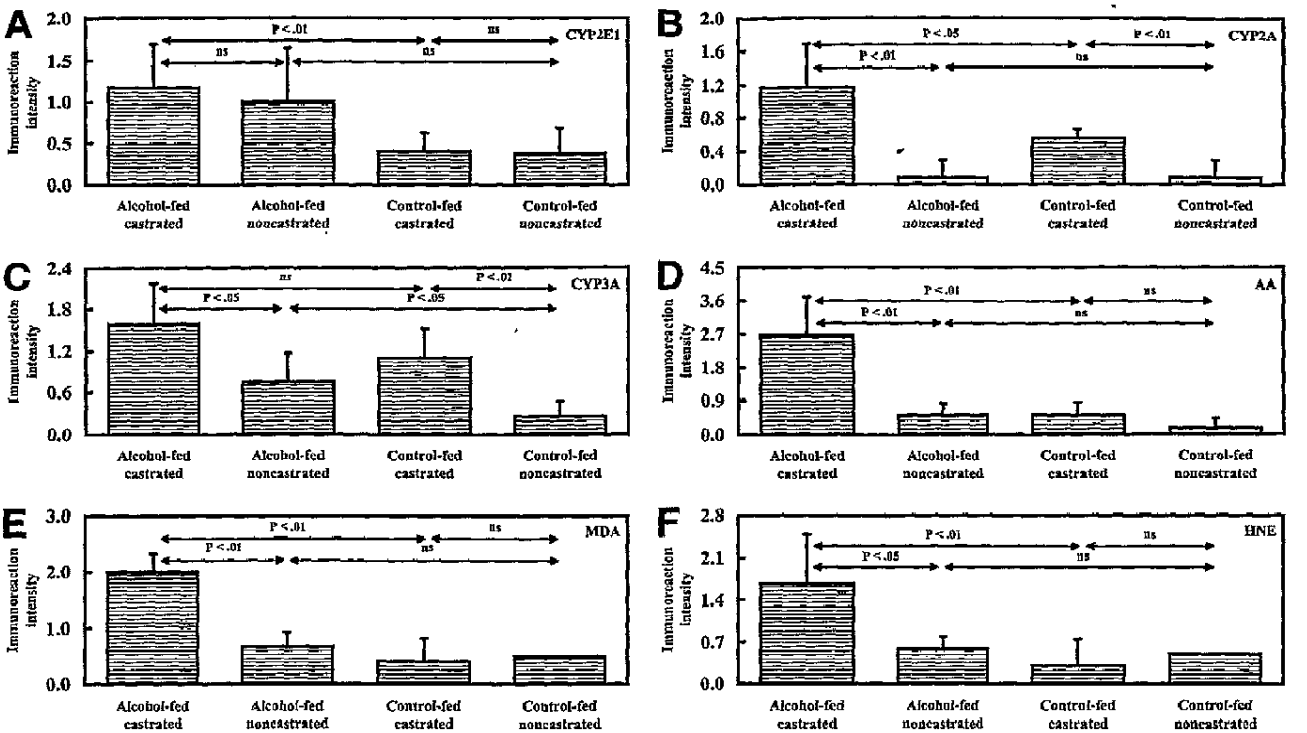

FIG. 3. Comparison of the relative intensities of CYP enzymes and aldehyde adducts in the castrated and noncastrated micropigs fed either with the ethanol-containing diet or with the control diet for 12 months. Values indicate means ± SD. Figures show the immunoreaction intensities for CYP2E1 (A), CYP2A (B), CYP3A (C), AA adducts (D), MDA adducts (E), and HNE adducts (F).

castrated control or ethanol-fed animals than in their noncastrated counterparts suggesting hormonal regulation for the expression of these cytochromes.

Despite the fact that blood ethanol levels were similar between the castrated and noncastrated micropigs during the present experiment, unique patterns of cytochrome responses to ethanol were noted between these groups. CYP2E1 induction by ethanol feeding was more pronounced in the castrated animals. The induction of CYP2E1 by ethanol has been previously well established and suggested to be responsible for a variety of toxic effects of ethanol in the liver and in other tissues.[3] The present findings on the co-occurrence of CYP2E1 induction and the generation of reactive aldehydic products is in accordance with previous findings from ethanol-fed rats and supports an association between CYP2E1 induction and liver pathology.[12,16,23,24] The correlation between hepatic CYP2E1 and aldehyde adducts also supports a role for CYP2E1 in the generation of these potentially toxic aldehydic species. Previous findings on the formation of protein adducts with AA, hydroxyethyl radicals, and CYP2E1 enzyme itself have indicated that CYP2E1 works in an environment that is exposed to high concentrations of AA.[25,26] Increased hepatic content of CYP2E1 together with its enhanced catalytic activity on ethanol consumption may further sensitize hepatic tissue to the toxic effects of AA and lipid peroxidation. Interestingly, previous studies have shown that inhibition of CYP2E1 activity may lead to clinical improvement of alcohol-induced liver injury.[27] The role of CYP2E1 induction in alcohol-induced liver disease has also

TABLE 2. Correlations Between Different Cytochrome Enzymes and Protein Adducts in Noncastrated Micropigs After 12 Months of Ethanol or Control Feeding

	CYP2A		CYP3A		CYP2E1		AA		MDA	
	r_s	n	r_s	n	r_s	n	r_s	n	r_s	n
CYP3A	0.279	12	—	—	—	—	—	—	—	—
CYP2E1	0.227	12	0.468	12	—	—	—	—	—	—
AA	0.513	12	0.775*	12	0.565	12	—	—	—	—
MDA	0.400	12	0.279	12	0.227	12	0.513	12	—	—
HNE	0.645†	12	0.288	12	0.342	12	0.444	12	0.645†	12

Abbreviation: r_s, Spearman rank correlation coefficient.
*$P < .01$.
†$P < .05$.

TABLE 3. Correlations Between Different Cytochrome Enzymes and Protein Adducts in Castrated Micropigs After 12 Months of Ethanol or Control

	CYP2A		CYP3A		CYP2E1		AA		MDA	
	r_s	n	r_s	n	r_s	n	r_s	n	r_s	n
CYP3A	0.865*	11	—	—	—	—	—	—	—	—
CYP2E1	0.735†	11	0.595	11	—	—	—	—	—	—
AA	0.599	11	0.359	11	0.733†	11	—	—	—	—
MDA	0.609†	11	0.500	11	0.851*	11	0.867*	11	—	—
HNE	0.342	11	0.158	11	0.460	11	0.732†	11	0.800‡	11

Abbreviation: r_s, Spearman rank correlation coefficient.
*$P < .001$.
†$P < .05$.
‡$P < .01$.

TABLE 4. Correlations of Cytochrome Enzymes and Protein Adducts With Sex Hormones in Micropigs After 12 Months of Alcohol or Control Feeding

		CYP2A		CYP3A		CYP2E1		AA		MDA		HNE	
Group	Hormone	r_s	n	r_s	n	r_s	n	r_s	n	r_s	n	r_s	n
Noncastrated animals	17-β-EST	−0.414	12	−0.210	12	−0.687*	12	−0.511	12	−0.473	12	−0.375	12
	Testosterone	−0.414	12	−0.510	12	−0.761†	12	−0.186	12	−0.296	12	−0.307	12
	17-β-EST/Testosterone	0.177	12	0.676*	12	0.313	12	0.723†	12	0.355	12	0.375	12
Castrated animals	17-β-EST	0.807†	10	0.551	10	0.689*	10	0.705*	10	0.687*	10	0.539	10

NOTE. The ratio of 17-β-EST/Testosterone was calculated only for those animals with detectable testosterone levels.
Abbreviation: r_s, Spearman rank correlation coefficient.
*$P < .05$.
†$P < .01$.

been suggested by recent findings indicating that the assessment of CYP2E1 content in paraffin-embedded liver samples could also aid in the diagnosis of heavy drinking.[28] However, based on the present results, it would appear that in the early phase of liver disease CYP2E1 levels may remain relatively low despite histopathological signs of progressive liver disease.

The present data show correlations between the hepatic contents of other CYP forms, CYP2A and CYP3A, and the appearance of aldehyde adducts and worsening of liver histopathology in castrated experimental micropigs. CYP3A induction after ethanol treatment has been recently reported also in rats.[14,15] The CYP3A protein, which is quantitatively the most abundant component of hepatic P450s and which has a variety of substrates, has previously been known primarily for its role in the activation of other types of hepatotoxins than ethanol, including acetaminophen, benzo(a) pyrene, and aflatoxinB1.[24] In addition, the present study shows, for the first time that hepatic CYP2A content may also be increased as a result of experimental ethanol treatment of castrated animals. Previous studies have shown CYP2A induction by cocaine administration.[17] Interestingly, ethanol is known to aggravate the hepatic injury produced by cocaine.[29] CYP2A may actually be a major catalyst of xenobiotic metabolism in damaged liver.[30] Taken together, the above data indicate a generalized induction of CYPs by ethanol, which may in turn be associated with a variety of adverse effects reported previously as a result of the generation of reactive aldehydes and enhanced oxidant stress,[31-35] including stimulation of fibrogenesis[36,37] and malignant transformation.[24]

In light of previous studies indicating a greater susceptibility of women for alcohol-induced liver injury,[3,38,39] it should be noted that in the present experimental model serum testosterone and 17-β-EST levels were associated with the amount of CYP enzymes, in particular with CYP2E1. Although castration abolished testosterone levels, it is of interest that chronic ethanol feeding had the feminizing effect of significantly increasing 17-β-EST in the castrated animals. Although precise mechanisms cannot be inferred, the present data suggest that hepatic levels of CYP2E1, AA, and MDA are regulated in part by deficiency of testosterone and/or relative increase in the ratio of 17-β-EST to testosterone. CYP2E1 increased in response to ethanol in both groups, but the increase was significant only in the castrated animals. The castrated experimental animals were found to present markedly more abundant amounts of CYP2A and CYP3A in the liver indicating that a premature (juvenile) metabolic phenotype, as induced by castration, may favor liver damaging processes. The observation that CYP2A was significantly induced by ethanol only in the castrated animals indicates that CYP genes may also exhibit sex-specific expression patterns as a result of toxic stimuli.[37] In fact, overexpression of CYP2A has previously been observed in female mouse livers suffering from chemically induced hepatomas.[40]

To conclude, the present finding of the greatest intensities of various CYP forms in hepatic tissue in castrated ethanol-fed micropigs that also had a clear evidence of oxidant adducts and more severe histopathology in the same vicinity support the view of an interactive role for these epitopes in the pathogenesis of alcohol-induced liver disease. This study also shows a correlation between the female metabolic phenotype and induction of these mediators of alcohol-induced liver disease. Although, the present micropig animal model was previously shown to develop comparable progressive histopathological features of alcohol-induced liver disease as described for humans, it remains to be established whether such findings could also be implicated in human patients with excessive ethanol consumption.

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